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13. ABSTRACT (Maximum 200 Words)

Cryptophycins are a potent, tumor-selective class of tubulin-binding antimitotic anti-cancer agents with excellent activity against MDR cancers. In order to develop these promising compounds into useful chemotherapeutic agents, it is necessary to obtain detailed information about the binding domain of the cryptophycins on tubulin. We plan to map the cryptophycin binding site through photoaffinity labeling studies. Toward this goal we have synthesized and studied the activity of a C16 side chain benzophenone photoaffinity analogues as well as a C10 azido analogues of cryptophycin-24. We have prepared a radioactive photoaffinity analogue and have initiated photolabeling studies. In addition, we are reporting an improved synthetic route to achieve the synthesis of diverse analogues in a more efficient way.

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Cryptophycin, microtubules, tubulin, azido analogues

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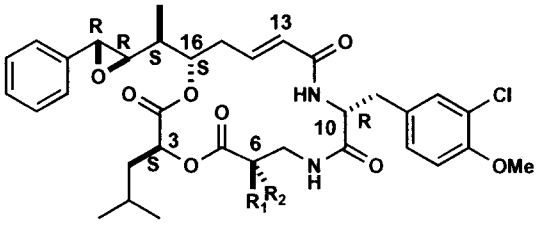
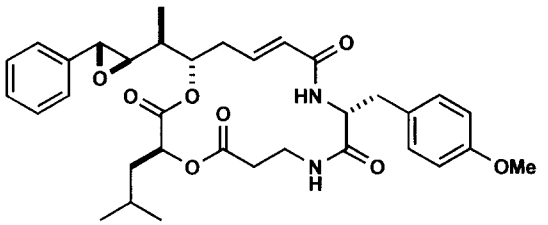
Introduction

Cryptophycins, isolated from blue-green algae (*Nostoc* sp.), are a new and potent tumor-selective class of tubulin-binding antimitotic agents[1] that show excellent activity against MDR cancer cell lines and were exceptionally active against mammary derived tumors.[2,3] Cryptophycin-1 (**1**, Fig. 1) is the major cytotoxin in *Nostoc* sp.[4,5] and displays IC_{50} values in the pM range. Of special importance is the reduced susceptibility of the cryptophycins to P-glycoprotein mediated multiple drug resistance in comparison to vinblastine, colchicine, and paclitaxel. A structurally related compound cryptophycin-24, (**2**, Fig. 1, also named arenastatin A), isolated from the Okinawan marine sponge *Dysidea arenaria*[6] and later from *Nostoc* sp. strain GSV 224,[7] is also a potent inhibitor of tubulin polymerization. Cryptophycins are one of the best recent lead in the search for anticancer therapies. Formal and total syntheses of the cryptophycins have been published by several groups.[8-14] Also, a multitude of SAR studies of these molecules have been reported.[2,3,15,16]

Fig. 1: Structures of cryptophycins



R_2 = H, Cryptophycin-1 or A (1)
R_2 = CH_3, Crytophycin-52 (3)



Cryptophycin-24 (2)
or Arenastatin A

Although relatively little is known about the interactions of cryptophycins with tubulin, it is believed that the cryptophycins may interact in a manner different from those of other tubulin-binding antimitotic agents.[17-19] For the development of these promising compounds into useful chemotherapeutic agents, detailed information about the binding domain of the cryptophycins is essential.[20] Hence, we planned to prepare analogues with affinity labels at the two aromatic rings of the cryptophycin molecule. The information obtained will be used to search for effective bioactive candidates for *in vitro* and *in vivo* testing. We have already synthesized and evaluated three C16 side chain benzophenone[21] and azido[22] analogues of cryptophycin-24. These molecules were found to be better tubulin binding agents than the parent compound cryptophycin-24. These photoaffinity analogues and are therefore candidates for photolabeling studies. A radioactive benzophenone analogue of cryptophycin-24 is now available and initial photolabeling experiments with this compound look promising.

We also developed a new synthetic method to prepare the Northern half of the molecule in seven fewer steps than reported before.

Body

According to the original statement of work proposed by Dr. Vidya Ramadas we fulfilled the goal of the tasks 1a, 1b, 1c, 2a, 2b, 3a, 3b, and 4a, 4b, as summarized below and in Schemes 1 to 9 and Tables 1 and 2 (Part A). Tasks 5a and 5b have been initiated, and we also carried out an additional study to improve our route for the synthesis of the Northern half of the molecule in order to expedite the process of synthesis of important analogs (Part B). During the switch of the postdoctoral grant from PI Dr. Ramadas to the current PI Dr. Yang, the appointment of Dr. Yang was delayed. Therefore, we have requested and obtained a no-cost extension for this project until April 30, 2006. During that time we intend to complete the photoaffinity labeling studies (task 5) and also re-synthesize compounds as needed for the affinity labeling studies.

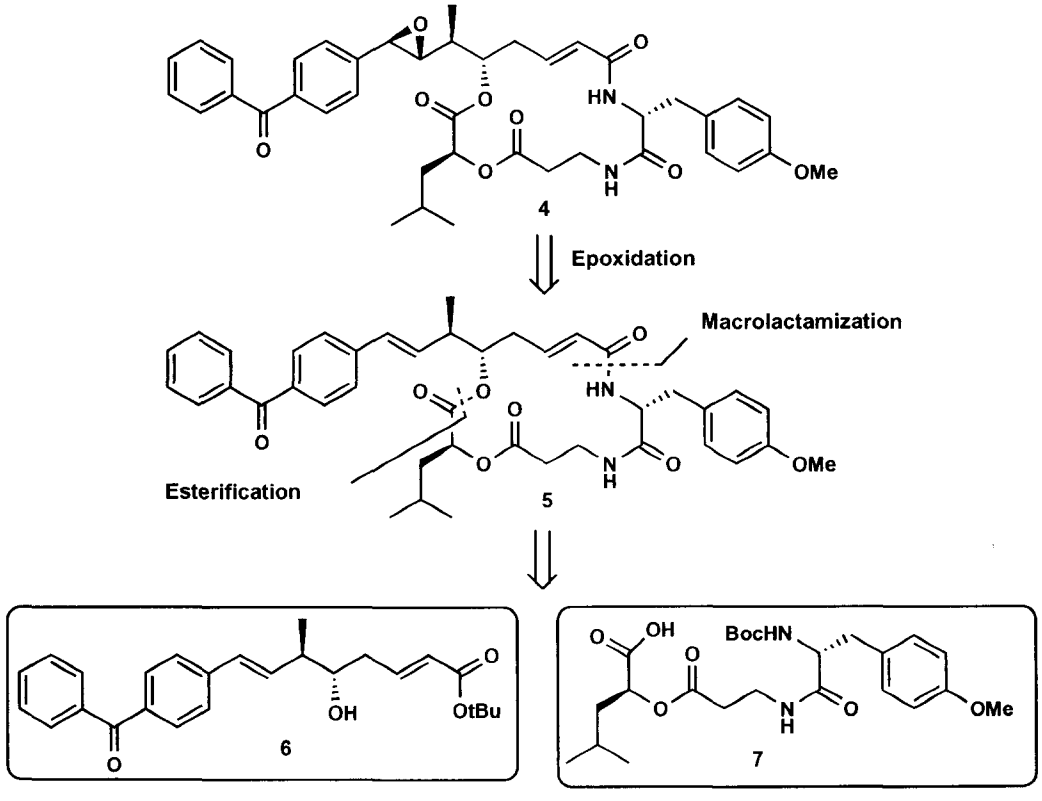
Part A. Summary of the work carried out during the first two years

Task 1. Synthesis of benzophenone derived cryptophycin-24 analogue, Month 1-12

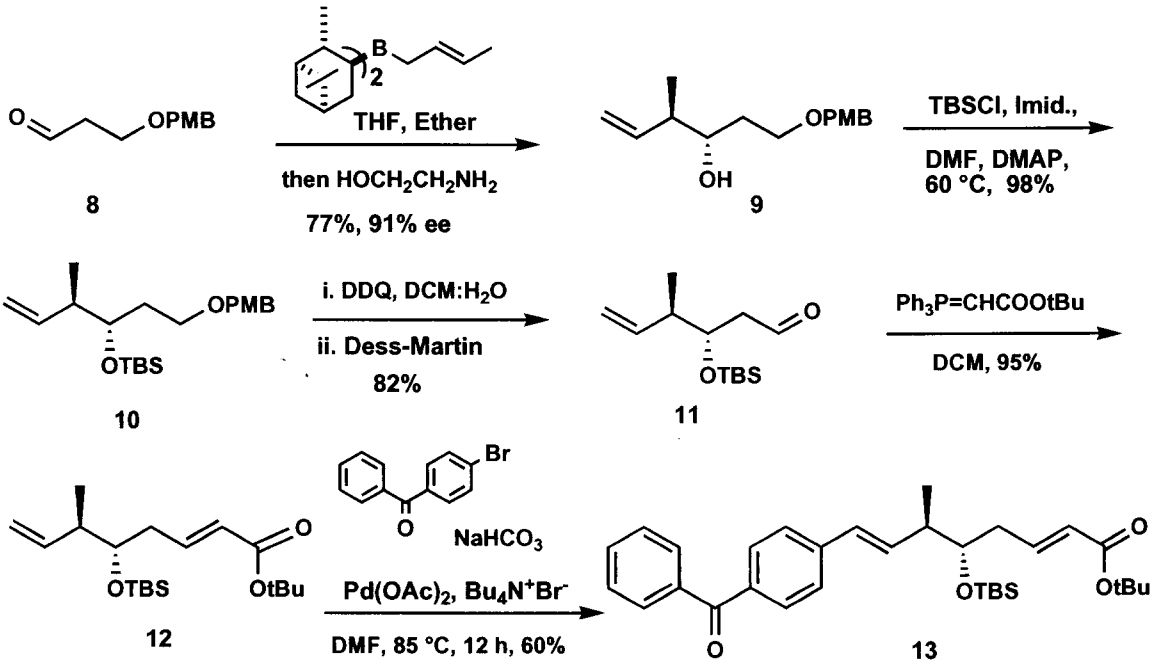
a. *Make large quantities of benzophenone derived octadienoate ester*
b. *Prepare the second important synthon, southern part of the molecule*
c. *Couple the two main synthons and convert it to the cryptophycin-24 analogue*

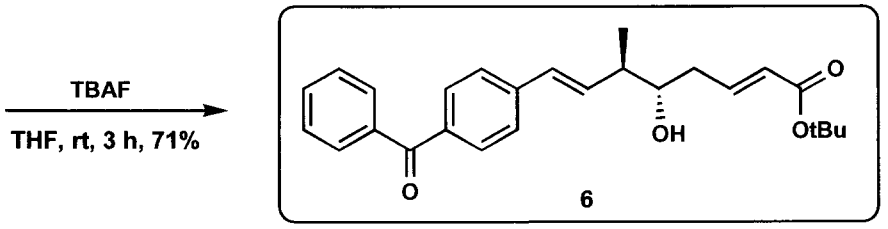
As shown below in Schemes 1-4, task 1, the synthesis of the benzophenone analogue **4** of cryptophycin-24, has been completed.

Scheme 1

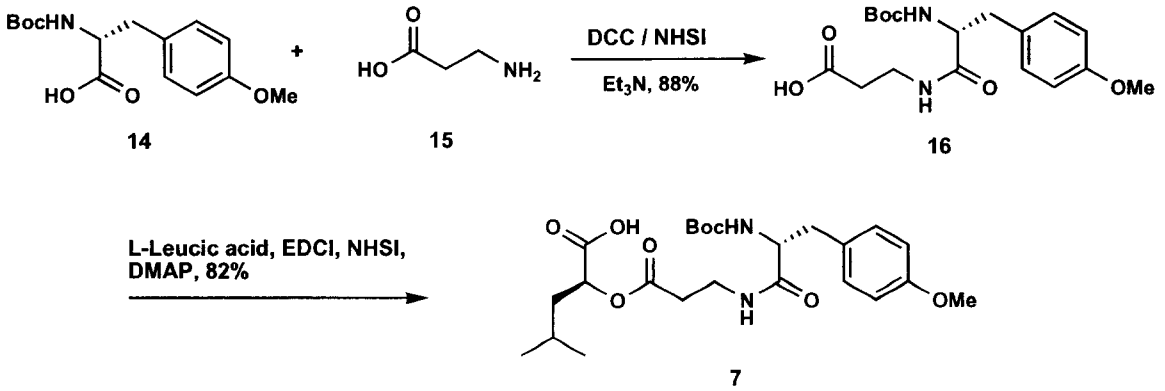


Scheme 2

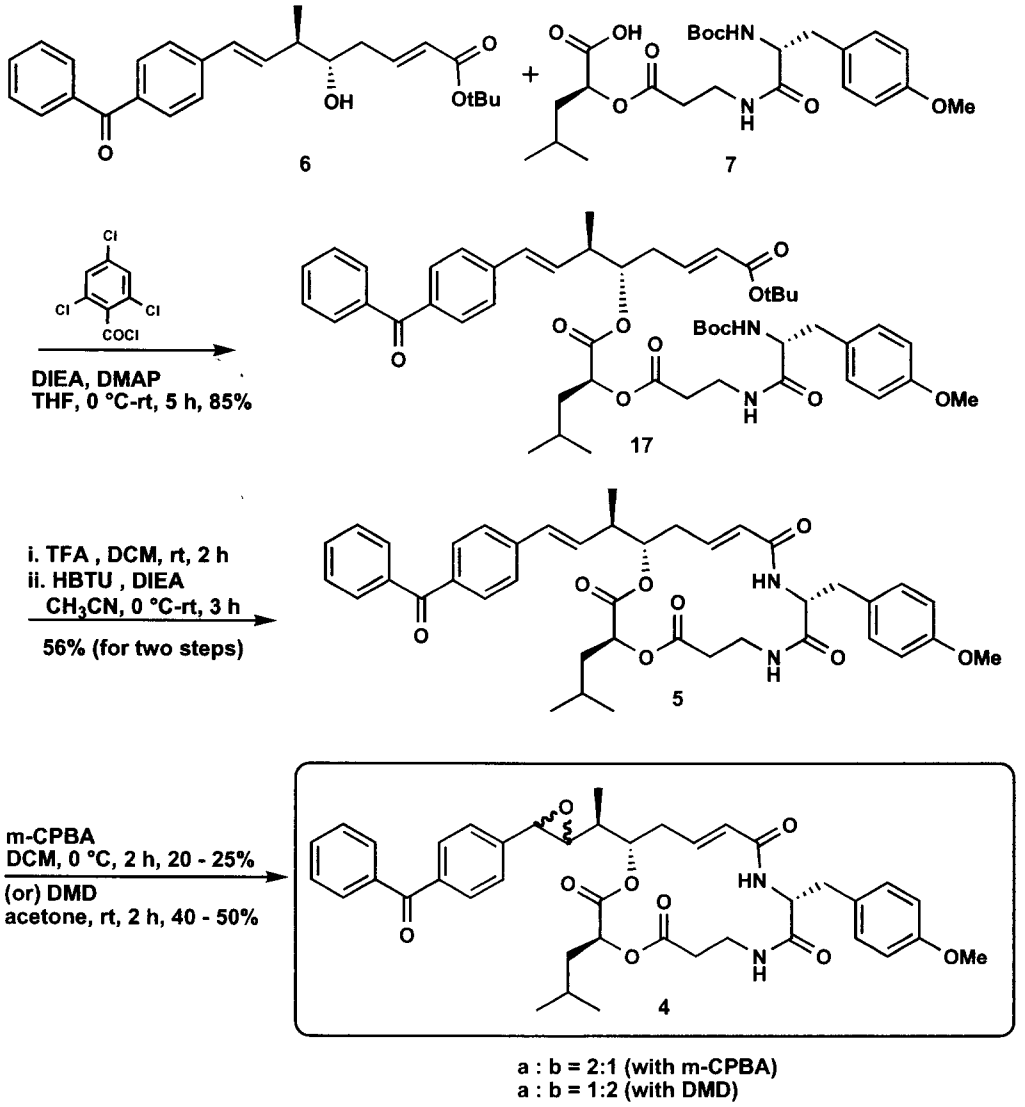




Scheme 3



Scheme 4



Task 2. Biological testing of benzophenone analogue of cryptophycin-24, Month 9-12

a. Tubulin assembly assay and cell culture assay will be performed on analogue
b. Determination of suitably active leads

As shown in Table 1 below, the benzophenone analogue **4**(β) was twice as active as the parent compound cryptophycin-24 (**2**) in the tubulin assembly assay and was therefore selected for radioactive synthesis and tubulin labeling.

Table 1. Biological Results

Compound	Tubulin Assembly IC_{50}, μM[a]	Cytotoxicity IC_{50}, nM[b]		
		MCF7	MCF7-ADR	HCT-116
1	3.7	0.003	0.013	0.027
2	15	0.13	0.164	0.285
4 (β)	7.4	0.078	70	1.1
4 (α)	>100	6.0	447	25.3

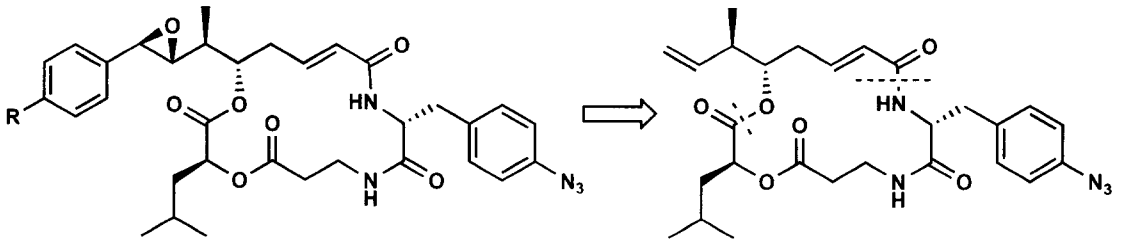
[a]Tubulin at 1.5 mg/mL was assembled at 37 °C for 15 min in the presence of PEM buffer, 0.5 mM GTP and 8% DMSO. Microtubules were pelleted and the protein remaining in the supernatant determined. The IC_{50} value is the concentration that reduces the amount of pelleted protein by 50%.
[b]The IC_{50} value is the concentration that inhibits the proliferation by 50% after 72h (MCF-7 and MCF7-ADR) or 24h (HCT-116) of cell growth.

Task 3. Synthesis of azido substituted cryptophycin-24 analogue, Month 13-24

a. Synthesis of octadienoate ester
b. Synthesis of azido derivative of cryptophycin-24

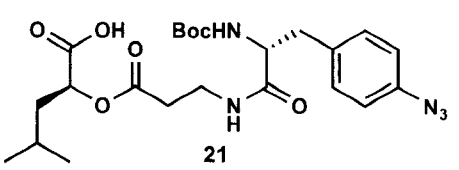
As shown below in Schemes 5-9, we completed the synthesis of three azido derivatives, **18a-c**, of cryptophycin-24.

Scheme 5



18a, R = H
18b, R = OAc
18c, R = OH

19



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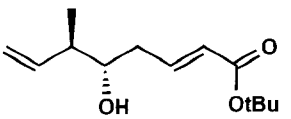

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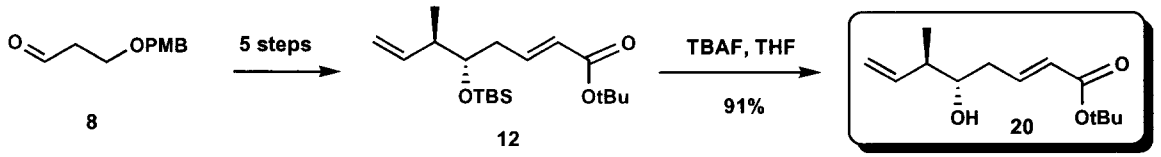
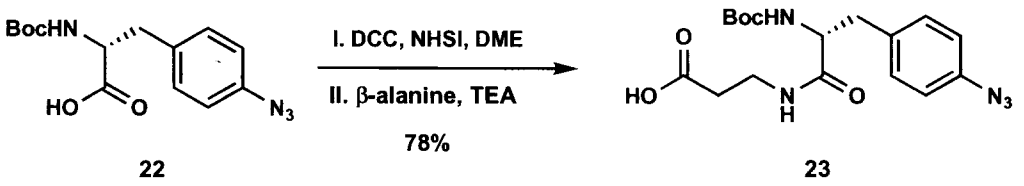
Scheme 6

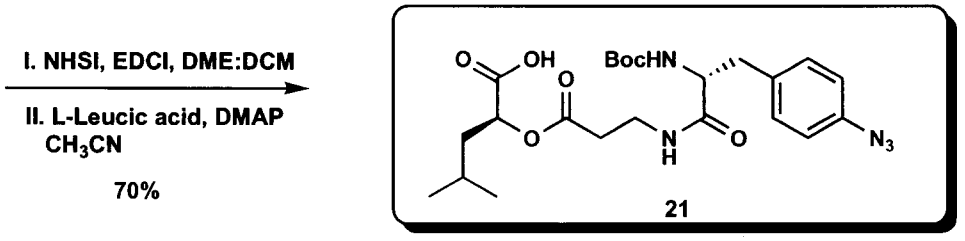


8 12 20

Scheme 7



22 23



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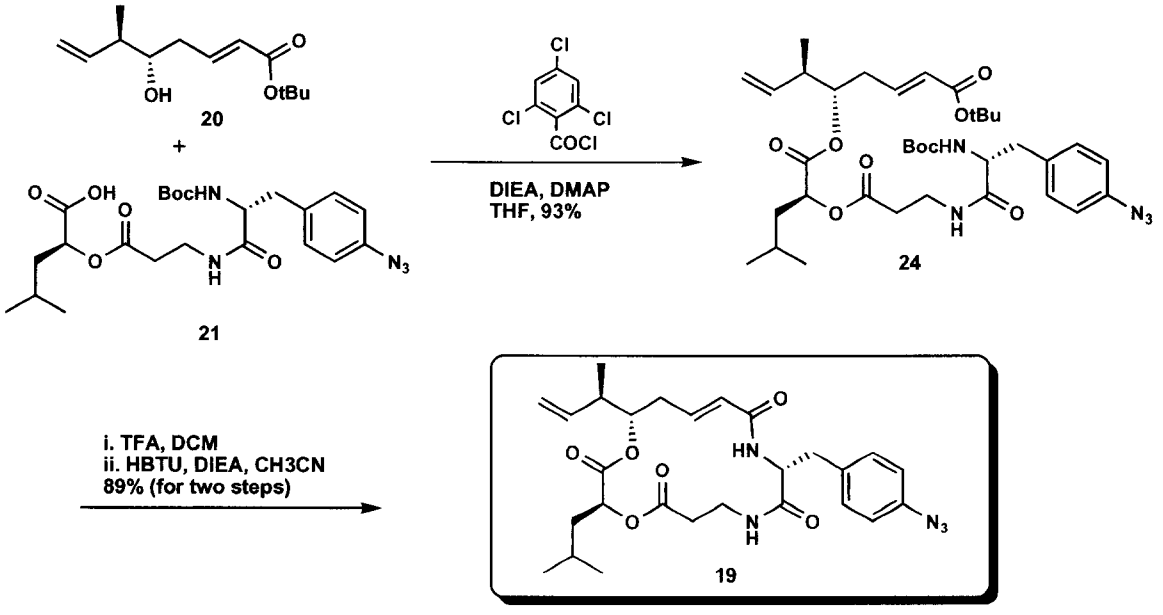
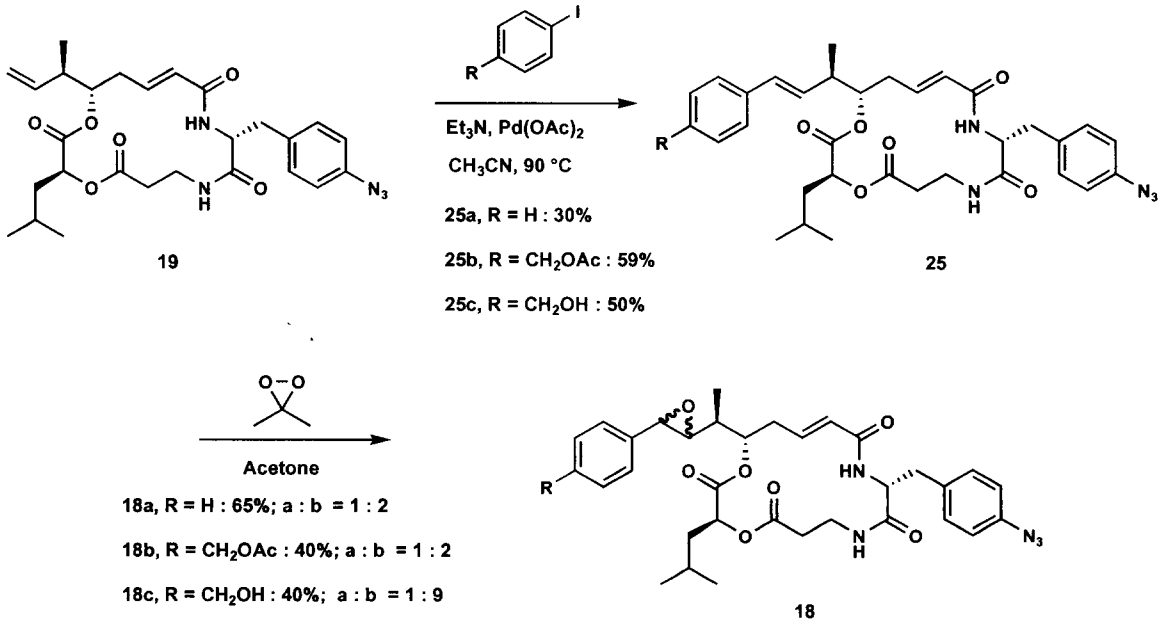
Scheme 8



Scheme 9

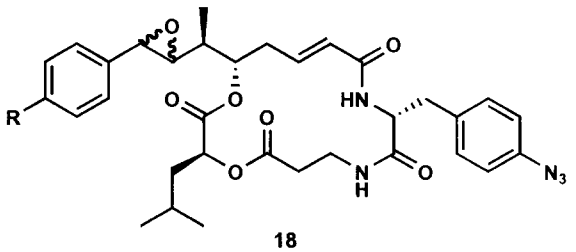


Task 4. Biological testing of azido analogue of cryptophycin-24, Month 21-24

a. Tubulin assembly assay and cell culture assay will be performed on analogue
b. Determination of suitably active leads

As shown in Table 2, the azido analogues **18a-18c** were similar in activity in the tubulin assembly assay compared to cryptophycin (**1**) and more active than cryptophycin-24 (**2**). These compounds are therefore excellent candidates for photolabeling studies.

Table 2. Biological Results.



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18a : R = H ; a : b = 1 : 2

18b : R = CH_2OAc ; a : b = 1 : 2

18c : R = CH_2OH ; a : b = 1 : 9

Compound	Tubulin Assembly IC_{50}, μM	Cytotoxicity IC_{50}, nM	
		MCF7	MCF7-ADR
1	3.7	0.003	0.013
2	15	0.13	0.164
18a	2	0.027	0.134
18b	4	not determined	
18c	4	not determined	

Task 5. Synthesis of tritiated analogues and start of labeling studies, Month 25-36

a. *Synthesis of tritiated analogues as determined by previous SAR studies on benzophenone analogue and azido analogue*
b. *Incubation of photoactivatable tritiated analogues with tubulin, photoactivation and cross linking*
c. *Digestion of tubulin protein and separation of peptides through various chromatographic methods, isolation of labeled peptide(s), analysis of peptides by FAB MS*
d. *Analysis of data and determination of the location of cryptophycin binding.*

Part B. Summary of the work from 2004 to current

Task 5a: *Synthesis of tritiated analogues as determined by previous SAR studies on benzophenone analogue and azido analogue*

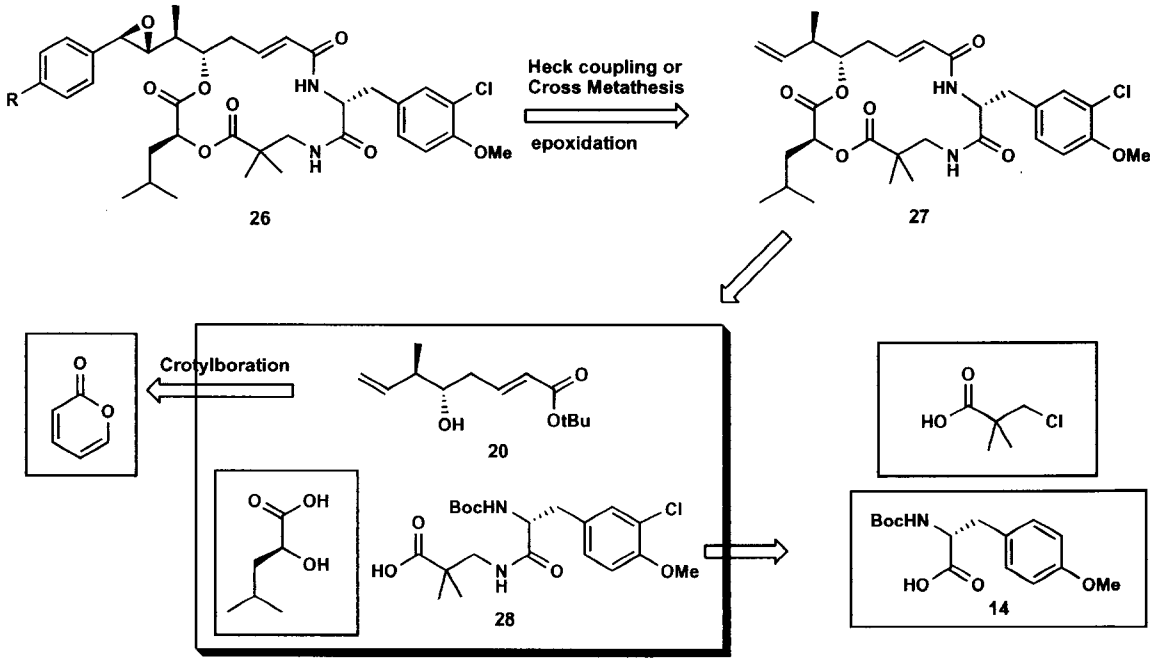
We prepared additional amounts of the benzophenone analogue **4**(β) and had the compound tritiated by a commercial company. Additional amounts of compound **18a** will also be prepared for radiolabeling after we have completed the labeling studies with benzophenone analogue **4**.

Task 5b: *Incubation of photoactivatable tritiated analogues with tubulin, photoactivation and cross linking*

Using the tritiated benzophenone derivative **4**(β), we have initiated a tubulin photolabeling study in order to identify the cryptophycin binding site. The basic experiment is to incubate the derivative with 20 μM tubulin in buffer and irradiate with a lamp of specific wavelength for a designated length of time. The protein is precipitated with 50% cold ethanol and washed several times with 50% ethanol. The precipitate is dissolved in 0.1 M NaOH, counted and used for protein determination. In the first experiment we tested the effectiveness of three different wavelengths in the procedure using 10 μM analogue. Radiation was for 10 min. The moles of analogue per mole of tubulin incorporated using 254 nm, 300 nm, and 350 nm radiation was 0.44, 0.32, and 0.11, respectively. In the next experiment we varied the concentration of analogue used and found increasing amounts of incorporation as the concentration was raised from 2.5 μM to 40 μM. Before trying to identify the sites on the protein that are modified we have several experiments to do to ensure ourselves that what we are observing is the result of specific labeling. The continuation of tasks 5 will be carried out during the no-cost extension time for the grant.

As shown below, we also developed an alternative, more effective scheme for the synthesis and re-synthesis of our photoaffinity labels (Scheme 10). The new method utilizes the four commercially available compounds shown in boxes: α-pyrone, 3-chloropivalic acid, and a D-tyrosine derivative. The main features of this route are that the route is seven steps shorter than previously reported ones and the use of only two protecting groups in the entire synthetic route, Boc and tBu.

Scheme 10

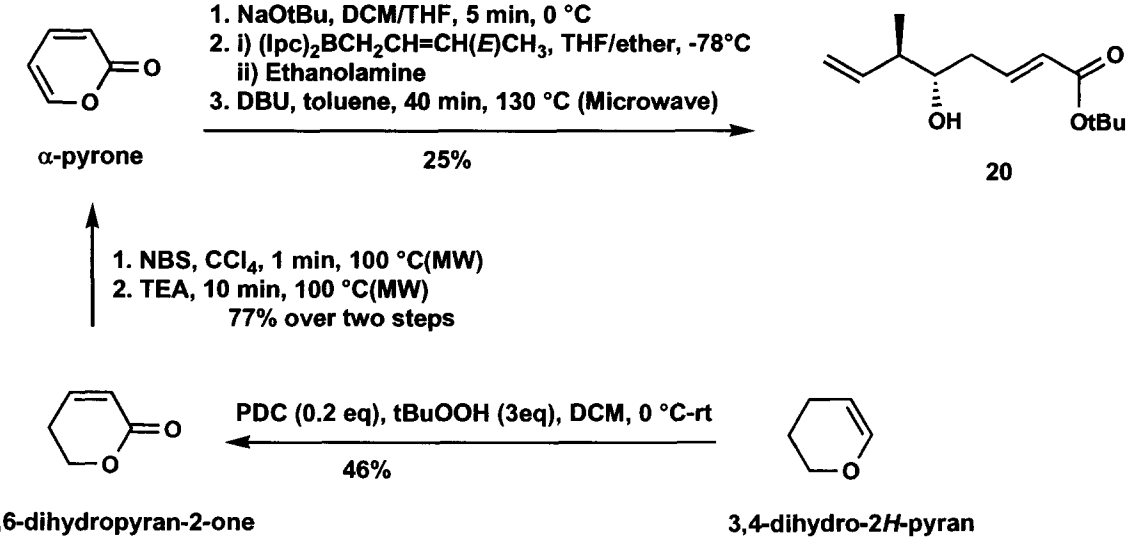


The synthesis of **20** can be accomplished from commercially available α-pyrone

in one continuous process in 25% overall yield (Scheme 11). This is a significant improvement over previous methods that provide the same precursor **20** in lower yields and in 8 to 10 steps. The aldehyde moiety in the α-pyrone was unmasked with NaOtBu and used for crotylboration. Double bond isomerization into conjugation with the carbonyl group with DBU provided building block **20**.[23]

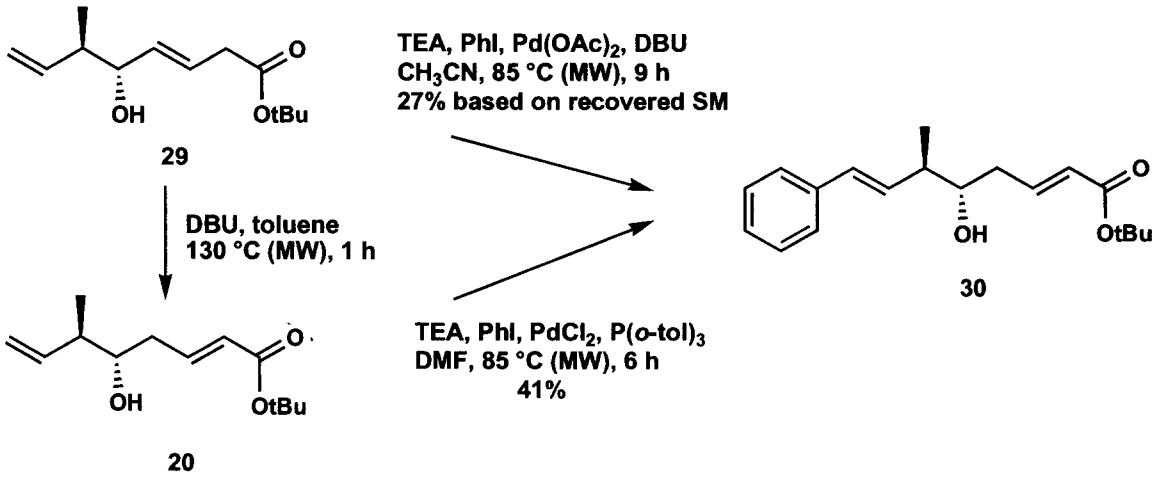
In order to circumvent the drawback of this method, related to the costs of the starting material, we successfully scaled up its synthesis[24,25] (Scheme 11). Both, 5,6-dihydropyran-2-one and 3,4-dihydro-2*H*-pyran are commercially available but the later is significantly less expensive. A known protocol exists to synthesize α-pyrone in one step from coumalic acid,[26] however, the equipment needed for the required decarboxylation reaction was not readily available to us and therefore we chose the two-step process.

Scheme 11



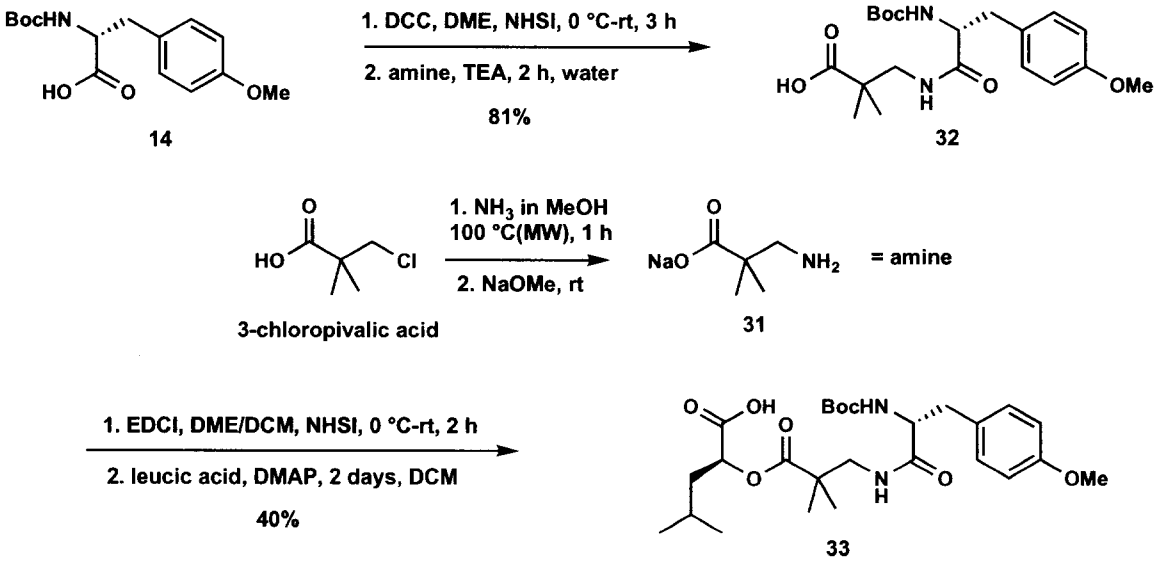
After achieving the synthesis of **20**, our plan was to attempt the subsequent Heck coupling (Scheme 12) with **29** (double bond out of conjugation) and without a protecting group. However, a mixture of all possible combination of products was observed. Therefore, double bond isomerization with DBU was carried out separately to yield **20**, which was used without purification for the Heck coupling reaction to furnish 41% of the desired octadienoate ester. However, the moderate yield of these reactions led us to consider attempting the Heck coupling at a later stage.

Scheme 12



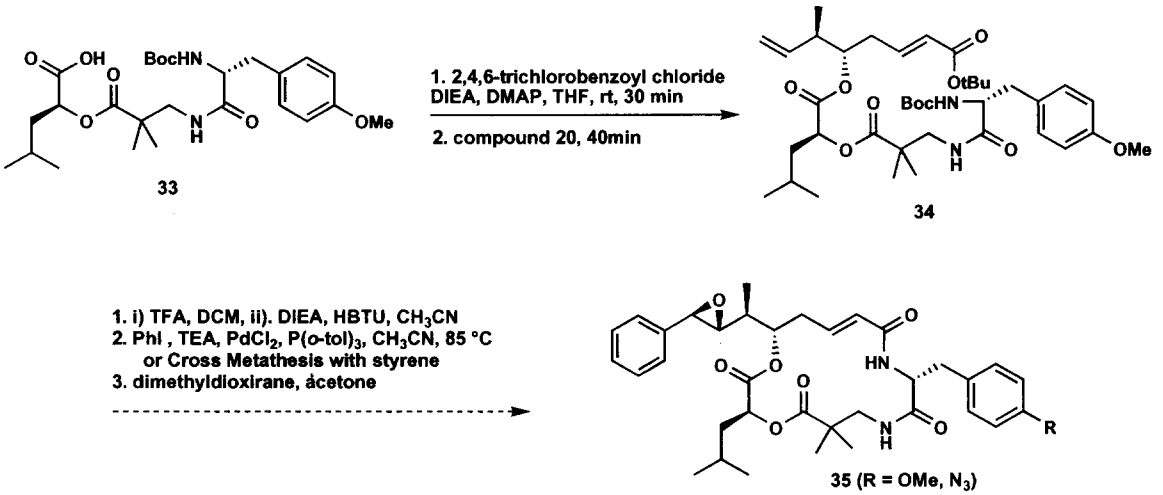
The synthesis of the Southern fragment was executed (Scheme 13) by following known methods except that the dimethylamino acid **31** (see cryptophycin-52, Fig. 1) was prepared from 3-chloropivalic acid and was utilized instead of β-alanine.

Scheme 13



The coupling of the two fragments **33** and **20** was realized using a traditional Yamaguchi reaction where the acid **33** is activated as the Yamaguchi chloride and reacted with alcohol **20**. The rest of the synthetic pathways to obtain final compounds **35** is currently being investigated.

Scheme 14



33

34

35 (R = OMe, N_3)

Key Research Accomplishments

- Total syntheses of two analogs, where a C10-azido and a C16-substituted aromatic side chains are attached, for elucidation of the tubulin binding site and structure-activity studies.
- Photoaffinity labeling studies of tubulin are being carried out with one of the analogues in its tritiated form in collaboration with Professor Richard Himes' laboratory in the Department of Molecular Biosciences.
- Establishment of a new, alternative chemical synthesis route to provide analogues in a more efficient way.

Reportable Outcomes

- Cryptophycin Affinity Labels: Synthesis and Biological Activity of a Benzophenone Analogue of Cryptophycin-24, R. Vidya, M. J. Eggen, G. I. Georg, R. H. Himes, *Bioorg. & Med. Chem. Lett.* **2003**, *13*, 757-760.
- Synthesis of Cryptophycins via *N*-Acyl-beta-Lactam Macrolactonization, R. Vidya, M. J. Eggen, S. K. Nair, G. I. Georg, R. H. Himes, *J. Org. Chem.* **2003**, *68*, 9687-9693.
- Cryptophycin Affinity Labels: Synthesis and Biological Evaluation of a C10 Aryl Azido Analog of Cryptophycin-24. R. Vidya, G. I. Georg, R. H. Himes, *Poster presented, 225th ACS National Meeting*, New Orleans, LA, United States, March 23-27, 2003.
- A Parallel Synthesis Demonstration Library of tri-Substituted Indazoles Containing New Antimutagenic/Antioxidant Hits Related to Benzydamine. S. Menon, H. Vaidya, S. Pillai, R. Vidya, L. Mitscher. *Com. Chem. High. Throu. Scr.* **2003**, *6*, 471-480.

Conclusions

We have achieved the total syntheses of a C16 benzophenone analogue (**4**) and three C10 azido analogues of cryptophycin-24 (**18a-c**). They were tested in a tubulin assembly assay, and for their cytotoxicity against MCF7, and MCF7-ADR breast cancer cell lines. The results showed that analogue **4** is twice as active as cryptophycin-24 in the tubulin assembly assay, twice as active as cryptophycin-24 toward MCF7, and 400 times weaker than cryptophycin-24 toward MCF7-ADR cell proliferation. Compound **18a** is seven times as active as cryptophycin-24 in the tubulin assembly assay, five times as active as cryptophycin-24 against MCF7, and as active as cryptophycin-24 against MCF7-ADR. Therefore, compounds **4** and **18a** are promising candidates to characterize the tubulin binding domain of cryptophycin by photoaffinity labeling. A radioactive analogue of benzophenone analogue **4** was prepared and was used for labeling studies. The preliminary results are promising. We are planning the continuation of these photolabeling studies with **4** and/or **18a** (in radioactive form), by digesting the tubulin protein and separating the peptides through various chromatographic methods. The isolated labeled peptide(s), will be analyzed by FAB MS and the analysis of data should determine the location of the cryptophycin binding domain. During the last year, we also significantly improved the methods for synthesizing photoaffinity analogues such as **4** and **18a** and other analogues of this family of compounds.

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